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Evaluation of cross-protection of a lineage 1 West Nile virus inactivated vaccine against natural infections from a virulent lineage 2 strain in horses, under field conditions

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1 **Title**

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4

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34 **Running title**

35 Horse WNV vaccine cross-protection field evaluation

36

37 **Abstract**

38 Although experimental data regarding cross-protection of horse WNV vaccines against
39 lineage 2 infections exist, their cross-protective efficacy under field conditions has not
40 been demonstrated. This study was conducted to evaluate the capability of an inactivated
41 lineage 1 vaccine (Equip[®] WNV) to protect against natural infections from the Nea
42 Santa-Greece-2010 lineage 2 strain. In total 185 WNV-seronegative horses in
43 Thessaloniki-Greece, were selected during two consecutive years (2011-2012). One
44 hundred and forty were immunized and 45 were used as controls. Horses were examined
45 for signs compatible with WNV-infection. Neutralizing antibody titers against the Greek
46 strain and the PaAn001/France lineage 1 strain were determined in immunized horses.
47 WNV circulation was detected during both years in the study area. It was estimated that
48 37% and 27% of the horses were infected during 2011 and 2012, respectively. Three
49 control animals developed clinical signs and WNV-diagnosis was confirmed. Signs

related to WNV infection were not observed in vaccinated animals. Non-vaccinated animals were associated with a $7.58 \pm 1.82\%$ higher chance of exhibiting signs compared to immunized ($P < 0.05$). Neutralizing antibodies raised against both strains in all immunized horses were detectable one month after the initial vaccination course. The cross-protective capacity of the lowest titer (1:40) was evident in 19 animals which were subsequently infected, and did not exhibit signs. Neutralizing antibodies were detectable until the annual booster, where strong anamnestic responses were observed ($GMTR_{lin.1} = 30.2$, $GMTR_{lin.2} = 27.5$). Results indicate that Equip[®] WNV is capable of inducing cross-protection in horses, against natural infections from a virulent lineage 2 WNV strain.

Keywords:

West Nile virus, Lineage 1, Lineage 2, Nea Santa-Greece-2010, Horse, Inactivated vaccine, Cross-protective immunity, Neutralizing antibody titer, Natural infections, Field evaluation

Introduction

West Nile virus (WNV) is a single-stranded RNA virus within the Japanese encephalitis virus serocomplex, which belongs to the genus *Flavivirus* (family *Flaviviridae*) (1). WNV is maintained in nature by enzootic transmission cycles between certain bird species and ornithophilic mosquitoes (2). Mosquitoes mainly belonging to the genus *Culex* can also act as bridge vectors, transmitting the virus to other animal species, including incidental hosts (3-6). Humans and horses are regarded as incidental (dead-end) hosts, as the virus titer developed in their blood is generally too low to infect mosquitoes (7). Nevertheless, WNV infection in susceptible hosts may eventually cause neurological disease (8). Regarding horses, the reported clinical signs may vary and these include fever, paraparesis or tetraparesis and ataxia, recumbency and behavioral changes, while

in many clinically affected horses muscle fasciculation and tremors are also present. It is expected that deaths will occur in a small percent of the affected animals (9-13).

Phylogenetic analyses of WNV strains isolated worldwide have resulted in the identification of 8 genetic lineages of the virus so far (14). Until 2004, only viral strains belonging to lineages 1 and 3 had been found in Europe. The majority of the strains isolated from European outbreaks belong to lineage 1 (15, 16). Lineage 2 includes strains from sub-Saharan Africa and Madagascar, and these had been so far considered of low-virulence (17). Such strains belonging to lineage 2 were isolated in Hungary (2004), in Austria (2008), as well as in Italy (2008) (16, 18). However, a virulent lineage 2 strain (Nea Santa-Greece-2010) was found to be responsible for the occurrence of 4 consecutive epidemic periods (2010-2013) in Greece, with neuroinvasive disease (WNND) cases in humans and horses during all these years (19-20). An amino acid substitution (H₂₄₉P) in the NS3 protein, absent from other closely related European strains, is suspected to be associated with the high virulence and neuroinvasiveness of the Greek strain (19). Enzootic transmission of the virus was detected once again in Central Macedonia, the epicenter of 2010 epidemic, during June 2014, using backyard chickens (21).

Experimental vaccinations in birds have been applied outside Europe (although bird vaccines against WNV are not commercially available) to a limited extent, especially in endangered bird species (e.g. in California condors) to protect them from fatal WNV infection, or in bird reservoir hosts (e.g. American crows and robins), aiming to reduce WNV viremia in them and prevent subsequent transmission of the virus to competent vectors (22-26). Regarding dead-end hosts, for humans only passive immunization (intravenous immunoglobulin or hyperimmune gammaglobulin administration) has been used to a limited extent for treatment of patients with WNND (27). No human vaccines against WNV are commercially available at this time, and as a result, active immunization of humans is not possible (28). In contrast, several inactivated and recombinant WNV vaccines for horses have been produced, evaluated and licensed in the USA. Specifically, two inactivated vaccines have been licensed and are being used at this time point in the USA; West Nile-Innovator[®] (Fort Dodge, IA, USA), and Vetera[®] WNV

(Boehringer Ingelheim Vetmedica, MO, USA). A recombinant vaccine with a Canarypox virus vector (Recombitek[®] Equine West Nile virus, Merial, GA, USA) is also marketed (19, 26, 29). It has been shown that all these immunologicals induce the production of WNV-specific neutralizing antibodies (NAbs) (30) and have proven to be very effective in protecting horses from meningoencephalitis in North America (31). Additionally, a DNA vaccine (West Nile-Innovator DNA[®], Fort Dodge, IA, USA) was approved by the USDA in 2005. Finally, a chimeric vaccine (PreveNile[™], Intervet, KS, USA) containing a strain of Yellow Fever virus (YFV-17D) was approved for marketing in 2006 by USDA, and was later remarketed as killed vaccine, under the name “EquiNile[™]” (19, 26, 32). All these immunologicals have been developed using lineage 1 WNV strains.

Along with the emergence of virulent lineage 2 WNV strains in Europe, two of the aforementioned vaccines, West Nile-Innovator [under the name Equip[®] WNV (Zoetis)] as well as Recombitek Equine West Nile virus [under the name Proteq West Nile[™], (Merial)] were authorized in 2011 and are being commercialized in European countries (33, 34). Concomitantly, questions arose whether these commercially available WNV vaccines for horses are effective in protecting them against virulent strains belonging to lineage 2, since both of them contain lineage 1 antigens, and their protection had not been extensively evaluated for other lineages. Previous experimental studies indicated that both of these vaccines can lead to the development of cross-protective immunity. Specifically, the recombinant vaccine “ALVAC[®]-WNV” (Merial) is capable of immunity induction in horses challenged with the goshawk-Hungary/04 lineage 2 strain (35). Another study which was conducted in mice immunized with the inactivated vaccine “Duvaxyn/Equip[®] WNV” has shown that it provided complete protection against challenge with the SPU93/01 lineage 2 strain (36). A more recent study was conducted in horses, showing that immunization with the Equip[®] WNV vaccine resulted in reduction of the number of viremic animals, the duration and severity of clinical signs of disease and mortality, following experimental infection with the virulent Nea Santa-Greece-2010 lineage 2 WNV strain (37). As a result, Equip[®] WNV was recently authorized also for lineage 2, although the duration of immunity has not been established for these strains

(33). Nevertheless, results regarding the evaluation of the cross-protection of these vaccines in field conditions are lacking.

It has been evidenced that, under experimental conditions, the effects of needle WNV inoculation in chickens might differ significantly from those of mosquito-borne natural infections (38). It has also been demonstrated that experimental WNV challenge in horses via needle inoculation, or mosquito feeding were not able to induce significant clinical signs (30). In addition, under experimental conditions, cell culture-adapted and passaged viruses are used as challenge strains. All these cultivation procedures might have consequent effects on the virulence of the viral strains. Consequently, field evaluation of viral vaccines is of utmost importance, in order to truly estimate the degree of cross-protection among different strains. In the present study we evaluated the capacity of the inactivated Equip[®] WNV vaccine to offer cross-protective immunity in horses against natural infections from the highly virulent Nea Santa-Greece-2010 lineage 2 WNV strain in field conditions.

Materials and methods

Animals

In total 185 mix-bred horses aged 5-18 years old were included in this 2-year study, which took place during the 2011 and 2012 epidemic periods in Greece. The horses belonged in 6 horse riding clubs in Central Macedonia the epicenter of the 2010 Greek epidemic. None of the horses had been previously exposed to WNV, as indicated by serological testing with competitive enzyme-linked immunosorbent assay (cELISA) and serum neutralization test (SNT) as described below. Specifically, serological testing was conducted twice; i) one week prior to the initiation of the immunizations in both years, and ii) at the time the first dose of the primary vaccination was conducted, for both years. The health status of each horse was determined prior to its incorporation in the study. Immunizations, blood samplings and clinical examinations of the animals were

performed by experienced veterinarians. A mixture comprised of oats, muesli and hay/alfalfa hay, was being administrated to the horses, and water was available ad libitum. Trained technicians were responsible for animal husbandry procedures.

Vaccine and immunization plan

The commercially available ready-to-use vaccine Equip[®] WNV (Zoetis, Louvain-la-Neuve, Belgium) was used in this 2-year study. This vaccine contains the inactivated lineage 1 WNV strain New York 1999/VM-2 (isolated from the brain of an infected horse during the 1999 epidemic period in New York, USA) formulated in MetaStim[™] oil emulsion adjuvant, consisting of Squalene, Poloxamer 401 (Pluronic[®] L121) and Polysorbate 80 (33). Vaccine lots 387BYC01L and 387BYA08A were used in 2011 and 2012, respectively. Each dose was administrated via a single intramuscular injection in the neck of the animals.

During June-July 2011 an initial double primary vaccination (two doses administrated 3 weeks apart) of 85 horses was performed (Fig. 1), while 33 horses were used so as to form the control group (Table 1). During May-June 2012, 79 of the aforementioned vaccinated animals received an annual booster immunization dose of the vaccine. Six of the original 85 horses were excluded during the second year for various reasons, e.g. they were moved out of the study area, or were euthanized due to causes irrelevant to WNV-infection. In addition, in May-June 2012, another 55 horses which were seronegative to WNV received a double immunization with the vaccine (Fig. 1). During this period, 21 of the 2011's control animals which were determined as seronegative were kept, and along with 12 additional seronegative horses, were used as naïve controls for the 2012 epidemic period (Table 1). The total number of control horses ($n = 45$) was intentionally limited to approximately 33% of the total number of horses used in the study for humane reasons. In each participating horse riding club the vaccinated and control animals were co-mingled and managed similarly.

189

190 Clinical examination and blood samplings

191 Physical and special neurological examination were being performed on each of
 192 the participating horses, at least one week prior to the initiation of immunizations, and
 193 until the end of the respective epidemic period. Monitoring was being performed
 194 regularly (every 5-6 days) for signs compatible with WNV infection, (e.g. anxiety,
 195 muscle fasciculation, head tremor, lip twitching, teeth grinding, ataxia, paresis, head
 196 shaking, etc.), along with any other abnormal conditions. Besides the evaluation for the
 197 presence of clinical signs due to WNV infection, horses were also being evaluated for
 198 local and systemic adverse reactions due to the vaccination. Clinical evaluations were
 199 done independent of knowledge of immunization status.

200 Blood was collected from all horses in 10 ml plain vacuum tubes at specific time
 201 points. For the animals participating from 2011, these time points were W (week) 0, W3,
 202 W7, W21, W34, W48, W52, W66, and W72 (Fig. 1). For the animals that participated in
 203 the study only during 2012, the respective time points for blood collection were W45(0),
 204 W48(3), W52(7), and W72(27) (Fig. 1). Numbers in parentheses indicate the exact week
 205 number in which samplings were being conducted from the horses that participated only
 206 in 2012, beginning from the week that these animals received the first dose of the vaccine
 207 (0). Numbers outside parentheses indicate the corresponding week number from the
 208 beginning of the study (2011). For example, W45(0) indicates week 0 for the horses
 209 participating in 2012 (conduction of the first immunization). Concomitantly, this is also
 210 week 45, counting from the day in which the first vaccine dose was administrated during
 211 2011 (W0).

212 Additionally, for the confirmation of diagnosis of WNV infection in horses with
 213 neurological signs, blood samples were drawn shortly after clinical signs were being
 214 noticed. Blood was allowed to clot and tubes were centrifuged ($3,000 \times g$, 10 min, 4 °C).
 215 Sera were transferred to clear 2 ml microcentrifuge tubes and stored at -80 °C until they
 216 were assayed.

217

218 Serological and virological testing

219 Sera obtained from all control horses after the end of both 2011 and 2012
 220 epidemic periods (W21 and W72(27) of the study, respectively) were tested for WNV-
 221 specific antibodies (indication of seroconversion), using a commercially available
 222 cELISA kit (ID Screen[®] West Nile Competition, ID.vet, Montpellier, France). This
 223 analysis was performed in order to confirm that the virus was circulating in the
 224 participating horse riding clubs, as well as to estimate the percentage of animals which
 225 were exposed to the virus during each epidemic period.

226 In order to confirm that the vaccine induced the development of cross-protective
 227 immunity, sera obtained one month after the completion of the double primary
 228 vaccination course from all vaccinated horses of both years (W7 and W52(7),
 229 respectively) were tested for the presence of NABs specifically directed against the Nea
 230 Santa-Greece-2010 lineage 2 strain, following an existing SNT protocol (39) with slight
 231 modifications. Briefly, after heat inactivation at 56 °C for 30 min, sera were two-fold
 232 serially diluted (1:5 to 1:2560, in duplicates in 96-well cell culture plates) in Dulbecco's
 233 Modified Eagle's Medium (DMEM; Invitrogen-Gibco, Groningen, The Netherlands), and
 234 50 µl of DMEM containing 100 TCID₅₀ (50% tissue culture infectious doses) of the Nea
 235 Santa-Greece-2010 strain were added. Controls, reference sera and back titration of the
 236 antigen were also included. After incubation of the plates at 37 °C for 1.5 h, 2×10^4 Vero
 237 cells in 100 µl of DMEM with 2% penicillin (100 IU/ml) and streptomycin (100 µg/ml),
 238 2% sodium pyruvate and 10% fetal bovine serum (Invitrogen-Gibco, Groningen, The
 239 Netherlands) were added to every well. Plates were incubated at 37 °C for 5 days and
 240 wells were examined under an inverted light microscope for evidence of viral cytopathic
 241 effects. The NAb titer of each serum was calculated as the highest serum dilution in
 242 which protection of the cell monolayer was observed. Sera were being considered
 243 positive if cells were protected at a dilution $\geq 1:10$.

Moreover, sera obtained from all primo-vaccinated horses (naïve horses which received the initial two-dose vaccination course) after the end of each epidemic period (November, W21 and W72(27) of the study) were also tested with the aforementioned SNT protocol and NAb titers were compared to the respective ones developed 1 month after the double primary vaccination (W7 and W52(7), respectively), so as to detect the occurrence of anamnestic humoral immune responses, indicative of natural infections.

In order to evaluate the levels and the duration of the produced NABs, 23 primo-vaccinated animals of the first year (2011) which were not exposed to the virus as indicated by the results of the aforementioned analysis (~40% of the total number of vaccinated animals which were determined not to be exposed to WNV during that year), were tested at samples obtained from the day of the first immunization (W0), until one month after the annual booster (W52). SNTs were used to determine the NAb titers against two WNV strains; the Nea Santa-Greece-2010 lineage 2 strain, as well as the PaAn001/France lineage 1 strain (kindly provided by Dr. Sylvie Lecollinet, UMR 1161 Virology, INRA-ANSES-ENVA, France).

For the confirmation of the diagnosis in control horses with clinical signs, the collected serum samples were tested for the presence of WNV-specific IgM antibodies, using a commercially available IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA; IgM WNV Ab Test, IDEXX-Istitut Pourquier, Montpellier, France), following the manufacturer's instructions. Furthermore, RNA was extracted from the sera obtained of the horses with neurological signs using the NucleoSpin® RNA virus kit (Macherey-Nagel, Düren, Germany. Extracts were examined using a WNV-specific, one tube real-time RT-PCR, using the primer pair WNPoUp (5'-TTTGGGAGATGGTGGATGARGA-3') and WNPoDo2 (5'-CCACATGAACCAWATGGCTCTGC-3'), at final concentration of 0.6 µM each, and the TaqMan probe WNPoProb2 (5'-FAM-TCTCTCTCTTTCCCATCATGTTGT-ZNA5-BHQ1-3' at a final concentration of 0.2 µM) targeting a 144 bp part of the nonstructural protein 5 (NS5) genomic region of WNV. The limit of detection was previously determined to be 1 TCID₅₀/ml (40). Amplification reactions were run in a

total volume of 25 µl using 5µl of RNA extract and 20µl of reaction buffer of a commercial RT-PCR kit (One step RT-PCR Qiagen, Hilden, Germany). The thermal cycling conditions were as follows; 50 °C for 30 min, followed by 95 °C for 15 min and 50 cycles in 2 steps: a) 95 °C for 30 sec (denaturation), and b) 60 °C for 40 sec (annealing and extension). The fluorescence levels were measured at the end of each cycle. The assay was performed using the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Analysis of data was conducted using the CFX™ Software (Version 3.0, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The effect of immunization on the presence of clinical signs was assessed with an odds ratio and a mixed model analysis.

The odds ratio analysis considered non-vaccinated animals as the control and vaccinated animals as the intervention (case) group. The odds ratio was calculated as follows (41):

$$OR = \frac{a}{b} \bigg/ \frac{c}{d} \quad [1]$$

where OR = odds ratio, a = number of vaccinated horses with clinical signs, b = number of vaccinated horses without clinical signs, c = number of non-vaccinated horses with clinical signs and d = number of non-vaccinated horses without clinical signs.

The significance of the odd ratio was assessed by the confidence interval which was calculated as follows:

$$95\%CI = e^{(\ln(OR) \pm 1.96 \cdot SE\{\ln(OR)\})} \quad [2]$$

where $95\%CI$ = 95% confidence interval, \ln = natural logarithm, SE = standard error and OR = odds ratio as in equation 1; the standard error was calculated as follows:

$$SE\{\ln(OR)\} = \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}} \quad [3]$$

where SE = as in equation 2 and a, b, c, d = as in equation 1.

In order to accommodate possible values equal to zero in the calculation of the odds ratio or its standard error, 0.5 may be added to all cells (42-43). An odds ratio significantly smaller than unity would suggest that intervention (i.e., immunization), is better than the control.

The mixed model analysis was based on the following model:

$$Y_{ijklm} = \mu + CY_i + RC_j + VS_k + A_l + e_{ijklm} \quad [4]$$

where Y = presence or absence of clinical signs for the l^{th} animal, μ = overall mean, CY = fixed effect of calendar year i ($i = 2011-2012$), RC = fixed effect of riding club j ($j = 1-6$), VS = vaccination status k ($k = 0$ for non-vaccinated and 1 for vaccinated animals), A = random animal effect reflecting the individual response of each horse and e = random residual.

Model 4 fitted a logit function to account for the binary nature of the trait (presence or absence of clinical signs). The outcomes of this model served as confirmation of the odds ratio analysis with the additional benefit of the quantification of the vaccination effect on presence of clinical signs, adjusted for all other factors included in model 4. The mixed model analysis was conducted with the ASReml software (44).

The two analyses, odds ratio and mixed model, were run once considering all animals and then a second time including only the infected horses.

NAb titers were used to calculate the geometrical mean titer (GMT) for each sampling time point and against each viral strain. The comparison of the GMTs against the two viral lineage antigens was performed at all sampling time points, using a paired two-tailed Student's t -test. A value of $P < 0.05$ was considered to be statistically significant. In order to estimate the strength of the anamnestic immune responses (e.g.

due to the booster) between two sampling points (w, z) and against the same lineage (x), geometrical mean titer ratio (GMTR) was calculated as follows: $GMTR_x = GMT_z / GMT_w$.

Investigation of the immunological similarity between the vaccine and the circulating viral strains

In an effort to interpret the immunological cross-reactivity between the NABs produced against the vaccine strain (New York 1999/VM-2, GenBank Acc. No. AF260967) and the lineage 2 strain circulating in the study area (Nea Santa-Greece-2010, GenBank Acc. No. HQ537483), we compared the identity of the Envelope (E) protein peptide sequences of the two strains. Furthermore, the respective peptide sequence of a lineage 2 strain isolated in South Africa (SA93/01, GenBank. Acc. No. EF429198) was included in these comparisons. Multiple alignments of E protein sequences were conducted using the MEGA v.6.06 software (45), and amino acid substitutions were visualized using BioEdit v.7.2.5 software (46).

Animal ethics

Animal studies have been performed in accordance to the International Guiding Principles for Biomedical Research involving animals, as issued by the Council for International Organizations of Medical Sciences. All horse owners gave their consent for the immunizations, blood sampling and serological testing prior to the commencement of the study. This study was performed in compliance with national guidelines and EU regulations, as well as by local Ethics Committees of the School of Veterinary Medicine, Aristotle University of Thessaloniki.

Results

348 WNV circulation in the study area

349 The presence and circulation of the virus in the study area was confirmed for both
 350 2011 and 2012, by studies conducted in captive sentinel chickens and mosquitoes, as
 351 already described (40, 47-48). Specifically, chickens were placed in cages in close
 352 proximity to the participating horse riding clubs, and exposed to mosquitoes throughout
 353 both epidemic periods, followed by serological and virological testing. Mosquitoes which
 354 were being collected throughout May-October of both years were also tested. Molecular
 355 characterization of the circulating viral strain during both 2011 and 2012 in chickens and
 356 mosquitoes confirmed that the virulent Nea Santa-Greece-2010 was the only strain
 357 detected in Central Macedonia (40, 47-48).

358

359 WNV natural infections in control and immunized horses

360 Serological testing of control horses during November of each year [W21,
 361 W72(27)] indicated that WNV circulated in all the participating horse riding clubs,
 362 during both 2011 and 2012 epidemic seasons. Specifically, 12 out of 33 control animals
 363 of 2011 (36%) and 9 out of 33 control animals of the 2012 period (27%) seroconverted to
 364 WNV as evidenced by cELISA testing (Table 1).

365 Comparative evaluation of NAb titers of sera obtained from all primo-vaccinated
 366 animals one month after the double vaccination and after the end of each epidemic period
 367 (November), indicated that anamnestic humoral responses (WNV infections) were
 368 evident in 32 of 85 primo-vaccinated horses of 2011 (38% of the immunized horses) and
 369 in 14 of 55 primo-vaccinated horses of 2012 (26% of the horses which received a primary
 370 immunization during the later year). Specifically, GMT increased from 1:67 to 1:1083
 371 (GMTR = 16.2, $\sim \log_2 4$ -fold increase). Natural infections, during 2012, of those horses
 372 that were exposed for a second consecutive year could not be determined directly by
 373 SNTs (due to the booster), but were calculated indirectly based on the respective
 374 percentages of the seroconverted control horses, as well as on the percentages of primo-

vaccinated horses in which anamnestic humoral responses due to infections were detected, for the two years. As a result, it is estimated that from the 52 horses which remained uninfected during 2011 and received an annual booster in 2012, 15 animals were subsequently infected from WNV during the second epidemic season (Table 1).

Combinatory analysis of all these results obtained from serological testing applied in control and vaccinated horses indicate in total 44 out of 118 horses (37%) were infected during 2011. The respective infection rate for 2012 was estimated to be 27% (Table 1). Infection rates between the horse riding clubs ranged between 18 and 60% for 2011 and between 18 and 47% for 2012.

Vaccine safety

Regarding the adverse reactions of the applied vaccine, only one out of 140 immunized animals (0.7%) developed a local reaction, on the site of the injection. This was a mild swelling which was developed after the second injection of the first year, and it was observed again in the same animal after the annual booster. During the aforementioned occurrences, resolution of the lesion was observed within a few days, without any interventions, and without other effects on the health of the animal.

Neutralizing antibody responses in immunized horses

One month after the initial double vaccination, NABs against the Greek lineage 2 strain were induced in all vaccinated animals with a GMT of 1:102 (titer range: 1:40 to 1:320). Briefly, in 67 out of the 140 vaccinated animals (47.9%; 95% CI: 39-56%), an intermediate neutralizing activity was observed (titers 1:40-1:80). Twenty-six of these animals developed a NAB titer of 1:40 and 41 animals were presented with a titer of 1:80. Higher neutralizing responses were observed in sera from the remaining 73 vaccinated animals (52.1%; 95% CI: 44-61%). Nineteen out of the 46 primo-vaccinated horses of

2011 and 2012 (41%) which were subsequently infected (32 and 14, respectively) as determined by SNT (Table 1), had a NAb titer of 1:40 against the Nea Santa-Greece-2010 strain, one month after the primary immunization course.

Moreover, application of SNT in 23 of the 53 primo-vaccinated horses of 2011 which were revealed to not be naturally infected indicated that GMTs against the lineage 1 strain were higher than the respective titer against the lineage 2 strain, at all sampling points. NABs in the sera of these animals were being consistently detected against the two viral lineage antigens, at all sampling points, and until the annual booster immunization (Fig. 2). However, paired *t*-test analysis revealed no significant differences in the GMTs against the two WNV strains in the sera of the 23 vaccinated horses, at all sampling points ($P > 0.28$). Specifically, analysis of the NAb titers raised against the two viral lineage antigens one month after the completion of the double primary vaccination course (i.e. on W7) indicated that the GMT for lineage 1 was 1:175 (titer range: 1:80 to 1:320), while the respective value for lineage 2 was 1:112 (titer range: 1:40 to 1:160). Individual NAb titers indicated that in 9 out of 23 animals (39.1; 95% CI: 20-61%) NAb titers were the same against both antigens. In 13 out of 23 animals (56.5; 95% CI: 35-76%) titers against the lineage 1 antigen were higher than the respective titer raised against lineage 2 by one serial dilution, while in one serum (4.4; 95% CI: 0.1-24%) a titer difference of 2 dilutions between the two viral lineage antigens was observed. Further comparison of NAb titers throughout the year indicate that those for lineage 1 were consistently higher than the respective values determined for lineage 2.

One month after the annual booster a strong titer increase was observed against both strains (Fig. 2). Achieved NAb titers were well above their initial peak (one month after the initial double primary vaccination). Specifically, $\geq \log_2 4$ -fold titer increase was observed in all cases, regarding the NAb titers against both PaAn001/France lineage 1 ($\text{GMTR}_{lin,1} = 30.2$) and Nea Santa-Greece-2010 lineage 2 ($\text{GMTR}_{lin,2} = 27.5$) strains. A similar degree of immunoreactivity (titer increase ≥ 4 two-fold serial dilutions) was also observed in the vaccinated animals which were naturally infected. In all these anamnestic immune responses NAb titers were determined to be $\geq 1:320$. The GMT for lineage 1 one

month after the annual booster (i.e. on W52) was determined to be 1:1894 (titer range: 1:640 to \geq 1:2560), whereas the respective value for the lineage 2 antigen was 1:722 (titer range: 1:320 to 1:1280). In terms of NAb titer differences between the two viral lineage antigens, it was indicated that in 15 out of 23 animals (65.2; 95% CI: 43-83%) NAb titers against the lineage 1 antigen were higher than the respective titer raised against lineage 2 by one serial dilution. In 7 out of 23 animals (30.4; 95% CI: 14-53%) titers differed by two serial dilutions, and in one serum (4.4; 95% CI: 0.1-24%) a titer difference of 3 dilutions was observed.

Clinical signs, confirmation of the diagnosis and cross-protective efficacy of the vaccine

None of the 140 vaccinated horses (0%) showed any clinical signs related to WNV infection during both epidemic periods. In contrast, 3 out of 45 control animals (7%) showed clinical signs due to WNV infection. The odds ratio, considering all animals, was 0.0432 (95% CI: 0.0022-0.8534). The fact that the CI did not cross 1 implies a statistically significant ($P < 0.05$) difference between vaccinated and non-vaccinated horses with regards to presence of clinical signs. The same analysis based on the infected animals only (61 immunized and 21 controls) returned an odds ratio of 0.0423 (95% CI: 0.0021-0.08562), implying that immunization was also beneficial for animals that were naturally infected.

More specifically, during the 2011 epidemic period, clinical signs were detected in one out of the 12 seroconverted control animals (August 13). During the 2012 epidemic period, 2 out of the 9 seroconverted horses of the control group showed clinical signs (August 25 and September 8, respectively). These signs included fever, weakness of hind limbs, ataxia, muscle twitching and tremors in all 3 affected animals. Diagnosis was confirmed, as WNV-specific IgM antibodies were detected in all of them by MAC-ELISA testing. WNV RNA was not detected (no Ct values obtained). Consequently, it was not possible to detect the virus in the obtained blood sera, since they were drawn after the initiation of the clinical signs and probably past the viremia stage. The 3 horses

received supportive treatment (dexamethasone, vitamin B complex supplements and phenylbutazone) that led to the resolution of clinical signs within a few days.

The effect of vaccination on preventing clinical signs was confirmed and quantified with mixed model analysis. Vaccination status had a significant effect on clinical signs, with non-immunized animals being associated with a $7.58 \pm 1.82\%$ ($P < 0.05$) higher chance of exhibiting signs compared to immunized animals. This value was derived from the analysis of all animals with mixed model 4. The value reflects the effect of immunization on the presence of clinical signs and describes the difference between the marginal means of vaccinated and non-vaccinated animals, adjusted for all other effects in model 4. The corresponding value from the analysis of infected animals only was $14.22 \pm 1.43\%$ ($P < 0.05$), suggesting that the vaccination effect was even stronger for naturally infected horses.

E protein amino acid sequence comparisons

For the interpretation of the reactivity between the NAbs raised against the vaccine strain and virus strains belonging to lineage 2, the immunological similarity of E protein peptide sequences was investigated. No differences were observed between the peptide sequences of the Greek and African lineage 2 strains. Sequence comparison of the lineage 1 vaccine strain with those of the lineage 2 strains, indicated 23 amino acid substitutions. Specifically, 3 of these substitutions were observed in structural domain I (DI; L₁₃₁Q, V₁₅₉I, and A₁₇₂S), 15 substitutions were identified in DII (E₅₅D, T₆₄S, K₇₁R, D₈₃E, R₉₃K, S₁₂₂T, I₁₂₆T, R₁₂₈W, T₁₂₉I, N₁₉₉S, T₂₀₅S, T₂₀₈A, T₂₁₀S, V₂₃₂T, and I₂₅₃V), 2 substitutions were present in DIII (L₃₁₂A, and A₃₆₉S), and 3 more substitutions were found in the transmembrane domain/stem region (K₄₁₃R, V₄₄₂I, and L₄₈₃M) (Fig. 3).

Discussion

Two doses of the vaccine, administrated 3 weeks apart in immunologically naïve horses resulted in the development of adequate cross-protective immunity against development of neurological signs due to natural infections from the Nea Santa-Greece-2010 lineage 2 strain during the following epidemic period, as indicated by the lack of occurrence of clinical signs in any of the immunized animals. Immunization using the aforementioned vaccine may not prevent horses from being infected from lineage 2 strains, but can reduce the number of viremic horses, the viremia duration and titer in the infected animals, the duration and severity of clinical signs and the mortality, as it has already been described (34, 37). In our case, although detection of severe cases in horses was effective due to the experience of the involved veterinarians, it is possible that mild clinical occurrences could have not been noticed. However, since no supportive treatment was required, the impact of these cases was insignificant. Adverse reactions due to the vaccine were minimal. Our findings confirm that although the majority of infections in horses were subclinical, a high percentage (14%) of the seroconverted non-vaccinated horses exhibited neurological signs. This is in agreement to a similar percentage (19%) of neurological manifestations-to-infections reported for this virus strain during the 2010 epidemic in Greece (49). Interestingly, slightly lower morbidity (10%) within infected horses has been reported for lineage 1 WNV strains (50-52).

Despite the use of adjuvants, long-term immunity is not a feature of inactivated vaccines. Although the duration of immunity for lineage 1 strains has been determined (12 months after the primary vaccination course) (33), relevant information for lineage 2 is lacking. Previous studies with Equip[®] WNV have indicated that immunized horses maintained NAb titers $\geq 1:100$ against lineage 1, for 5-7 months, as determined by plaque reduction neutralization test (PRNT) (53). In another study it was shown that neutralizing responses were maintained for 6 months after vaccination of immunologically naïve horses (54). In a more recent study, it has been demonstrated that NABs could be detected at samples obtained one year after the primary vaccination course of naïve horses, although a decline in neutralizing titers was observed (55). In our case, NABs against the lineage 2 strain were developed in all vaccinated animals (titer range: 1:40 to 1:320,

GMT = 1:102) one month after the initial double vaccination. Although a titer decline was observed through time, as evidenced by testing of vaccinated animals that were not infected (GMT < 1:100 on week 34), NABs were detectable until the annual booster. The lowest neutralizing response of 1:40 against the lineage 2 strain was observed in 41% of the primo-vaccinated horses of both years ($n = 19$) which were subsequently infected. The fact that these 19 horses did not exhibit clinical signs due to WNV infection indicates that NAb titers as low as 1:40 one month after the primary immunization course can be protective against natural infections from the Nea Santa-Greece-2010. It can be hypothesized that humoral immunity against lineage 2 lasts at least until W21, based solely on the GMTs against the lineage 2 strain which were >1:100 for these sampling time points, although individual titers >1:100 were detected until W34. GMTs against the PaAn001/France lineage 1 were higher than the respective titers against lineage 2, which is in agreement with other studies (36, 55). In our case comparison of GMTs against the two lineages revealed no significant differences. The applied immunization scheme resulted in development of adequate B-cell memory, as indicated by the strong responses observed after annual boosters and natural infections. Results regarding these responses are supported by a previous study, in which significant NAb titer increase ($\log_2 5$ -fold) against both lineages is described, and titers achieved were well above their peak observed after the initial vaccination (55). It has been previously demonstrated that horses immunized with this vaccine also developed antigen-specific cellular responses ($CD4^+$ and $CD8^+$ IFN- γ expression, cellular proliferation and IL-4 expression in $CD4^+$ PBMCs) (54), indicating that, besides humoral immunity, the vaccine induces T-cell responses, which might have an additional contribution to the cross-protection of the naturally infected horses.

WNV E protein is a major determinant of tropism and the primary target of NABs. Neutralizing epitopes have been identified mainly on DIII of E protein, and specifically, on residues 306, 307, 330 and 332 (56-58). Additional neutralizing epitopes have been identified on several residues of DI and DII, although the observed neutralizing activity for these regions is weaker (59). In our case, no changes were observed at residues S₃₀₆,

K₃₀₇, T₃₃₀ and T₃₃₂, which serve as major DIII neutralizing epitopes (56-58). However escape from neutralization has been associated with the L₃₁₂A substitution, which was present in “Nea Santa-Greece-2010”, as it is in several WNV strains (60). No changes were observed in residues W₁₀₁, G₁₀₆ and L₁₀₇, antigenic sites of the fusion loop located within DII (DII-FL, residues 98-109), which act as target of cross-NAbs among different species of the genus *Flavivirus* (61-63). Despite the L₃₁₂A substitution, the findings of the present study ultimately suggest that under field conditions, adequate cross-neutralization is capable of providing a high degree of protection.

Different WNV lineages, characterized by varying virulence and neuroinvasiveness co-circulate in Europe (63), and knowledge regarding the cross-protection is prerequisite. However, since outbreaks in horses were limited and unpredictable, immunizations have been performed extensively (26), regardless the degree of cross-protection between circulating and the vaccine strain. For the purpose of in-field evaluations of arbovirus vaccines, identification of the circulating strain comprises a necessity. In our case, it was not possible to detect the virus in the affected horses. This was anticipated, given that in horses WNV detection is hampered by the short viremia duration which precedes the onset of clinical signs (7, 49, 51). Therefore, WNV surveillance data from birds and mosquitoes, indicating that the only strain circulating during both years was the Nea Santa-Greece-2010 were utilized (46-48). Mixed model analysis seems to be a more accurate approach for in-field vaccine evaluations, as many factors are involved and should be taken into consideration. It was also possible to quantify the favorable effect of the immunization on the presence of clinical signs. Immunizations using inactivated lineage 1 vaccines can effectively protect horses from the development of neurological signs due to natural infections of virulent lineage 2 WNV strains. Since pathogenesis and antiviral immune responses against WNV in horses and humans are similar, our results could be of value in the future, for the possible evaluation of a candidate human vaccine.

Conflict of interest

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795 **Figure captions**

796 **Fig. 1.** Timeline of the immunizations and blood serum samplings performed in horses,
 797 for the evaluation of the cross-protective immunity offered from the inactivated vaccine.
 798 Black syringes indicate the double primary vaccinations; white syringe indicates annual
 799 booster vaccination. Arrows depict the time points of blood serum samplings. Weeks in
 800 which these samplings were conducted are displayed above the arrows. The two black
 801 arrows marked with asterisks (*) depict samplings performed one week prior to the
 802 initiation of the primary vaccinations of 2011 and 2012, respectively, in order to detect
 803 and select WNV-seronegative horses.

804 **Fig. 2.** Neutralizing antibody (NAb) geometrical mean titers (GMTs) of 23 immunized
 805 horses which were not infected, against the Nea Santa-Greece-2010 lineage 2 strain (grey
 806 curve, ◆) as well as the PaAn001/France lineage 1 strain (black curve, ▲). SNTs were
 807 performed in sera collected from W0 (time of the first dose of the double primary
 808 vaccination in 2011) and until W52 (i.e., one month after the annual booster
 809 immunization). Paired *t*-test analysis revealed no significant differences in the NAb
 810 GMTs against the two antigens, at all sampling points ($P > 0.28$). Range error bars
 811 encompass the range of the individual NAb titers against each antigen, and for every
 812 sampling time point. Geometrical mean titer ratio (GMTR) calculated for the annual
 813 booster against each strain is also presented.

814 **Fig. 3.** Alignment of the E protein amino acid sequences from the vaccine lineage 1 strain
 815 “New York 1999/VM2” (GenBank Acc. No. AF260967), the circulating lineage 2 strain
 816 “Nea Santa-Greece-2010” (GenBank Acc. No. HQ537483), and the South African
 817 lineage 2 strain “SA93/01” (GenBank Acc. No. EF429198) of West Nile virus. Dots
 818 indicate amino acid identities. The domains are indicated by bars, as explained at the
 819 figure legend. Investigation of the immunological similarity between the three peptides
 820 revealed no differences between the two lineage 2 strains. Comparison of the lineage 1
 821 (vaccine) and the lineage 2 peptide sequences of the E protein revealed 23 amino acid
 822 substitutions.

823 **Table 1.** Numbers of immunized and control horses which were included in the efficacy
 824 study during 2011 and 2012. Numbers and infection rates of horses per year, as well as
 825 numbers of horses which exhibited neurological signs due to WNV infection are also
 826 included in the table.

Horse group	2011		2012		
	IH (WNV-seronegative, primo-vaccinated)	CH (WNV-seronegative)	IH (WNV-seronegative, primo-vaccinated)	IH (primo-vaccinated in 2011 but not infected, received annual booster vaccine dose in 2012)	CH (WNV-seronegative of 2011 but not infected + WNV-seronegative, selected in 2012)
No. of horses per group	85	33	55	52	33 (21+12)
No. (%) of WNV naturally infected horses determined by cELISA and/or SNT	32/85 (38%)	12/33 (36%)	14/55 (26%)	ID: 15/52 (29%)	9/33 (27%)
No. (%) of WNV naturally infected horses with clinical signs, confirmed by MAC-ELISA	0/32 (0%)	1/12 (8%)	0/14 (0%)	0/15 (0%)	2/9 (22%)
Total No. (%) of infected horses per year	44/118 (37%)		38/140 (27%)		

827 IH, immunized horses; CH, control horses; cELISA, competitive enzyme-linked
 828 immunosorbent assay; SNT, serum neutralization test; ; MAC-ELISA, IgM antibody
 829 capture enzyme-linked immunosorbent assay; ID, indirect determination

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